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Effect of 12-O-Tetradecanoylp[illegible]rbol-13-Acetate on the Morphology and Growth of C3H/10T1/2 Mouse Embryo Cells

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Effect of 12-*O*-Tetradecanoylphorbol-13-acetate on the Morphology and Growth of C3H/10T1/2 Mouse Embryo Cells[1]

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ABSTRACT

The effects of the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the morphology and growth properties of C3H/10T1/2 clone 8 cells were examined. The morphology of these cells was changed within 30 min following treatment with 0.1 μg of TPA per ml; they became smaller and refractile with long beady processes. Such changes were observed in both logarithmic and confluent cultures and lasted about 72 hr. Subsequent treatments were much less effective in inducing these changes. Scanning electron microscopy showed cell retraction and rounding as the most significant immediate effects of TPA treatment; many cells remained partially rounded 48 hr afterwards. Long-term surface modifications ascribable to TPA treatment were not detected. TPA had only minor effects on the growth of cultured C3H/10T1/2 cells in the presence of 10% fetal calf serum. Slight increases in plating efficiencies and saturation densities were generally observed in the presence of TPA but not with the related non-tumor-promoting compound phorbol. The cells grew slowly in 1% fetal calf serum and demonstrated serum batch-dependent alterations in their growth properties when exposed to TPA. Under conditions that produce doubling times of 70 hr or greater, TPA, but not phorbol, reduced the doubling time to about 50 hr. Saturation densities were also increased by TPA in 1% fetal calf serum. The effects of TPA on the growth of an oncogenically transformed variant of C3H/10T1/2 were quite different. While minimal effects of TPA were observed when transformed cells were treated in the presence of 10% fetal calf serum, TPA treatment in 1% fetal calf serum significantly inhibited cell growth.

INTRODUCTION

An increasing body of evidence suggests that the occurrence of neoplasia in humans and in experimental animals is due to a multistep process (see Refs. 1 and 4 for reviews). At least 2 steps have been identified in mouse skin experimental systems (1, 4). The first step, initiation, is irreversible, is produced by many different carcinogens, and may or may not result in cancer. The second step, promotion, is reversible and can be accomplished by repeated treatments with noncarcinogenic agents that facilitate the development of oncogenicity in cells that have been altered by previous contact with an initiator. The most potent tumor promoters known have been isolated from croton oil, an extract from the seed of the plant *Croton tiglium* (12). The active components of croton oil are phorbol esters, the most potent of which is TPA[5] (12).

Recently, initiation and promotion have been demonstrated in cell cultures. The first observations of this were reported by Lasne *et al.* (16, 17), who examined the effect of TPA on the transformation of rat embryo fibroblasts by benzo(*a*)pyrene (16, 17) and 7,12-dimethylbenz(*a*)anthracene (17). Although the authors claim to have demonstrated initiation and promotion in cell culture, alternate interpretations of their data are possible. High rates of spontaneous transformation were observed in their experiments, and the application of TPA sometimes increased the spontaneous transformation rate to a level comparable to that observed in cultures treated with both initiators and promoters.

Initiation and promotion have also been reported in the transformation of C3H/10T1/2 Cl 8 mouse embryo cells. Spontaneous transformation in these cells is rare (23, 28), and unambiguous results have been obtained. TPA has been found to promote the transformation of C3H/10T1/2 cells initiated previously by X-rays (15), ultraviolet radiation (25), or polycyclic aromatic hydrocarbons (24).

Numerous other cell culture studies have been conducted to examine the effects of TPA and other tumor promoters on a variety of cell types. Cells appear to respond in a pleiotropic fashion to the presence of TPA. The results of such studies (see Refs. 7 and 32 for reviews) often suggest that tumor promoters enhance the expression of cellular properties that are frequently associated with the neoplastic state. Evaluating the significance of the majority of such studies is difficult since the promotion of chemically initiated transformation has not been reported in most of the cell culture systems utilized. One can thus question whether the majority of such observations are relevant to the phenomenon of tumor promotion.

Relatively little work has been published concerning the biological effects of tumor promoters on C3H/10T1/2 Cl 8 cells (see Ref. 23 for a review), the transformation of which is promoted by TPA. We report here studies examining the effects of TPA on numerous properties of C3H/10T1/2 Cl 8, several of which are known to be altered in oncogenic transformation: morphology (28); cell surface morphology (21); saturation density (28); and the ability of cells to grow in low (1%) FCS (2). The effect of TPA on a transformed variant of C3H/10T1/2 Cl 8 is also examined.

MATERIALS AND METHODS

Chemicals. TPA and phorbol were obtained from Consolidated Midland Co., Brewster, N. Y. Stock solutions of TPA

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[5] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FCS, fetal calf serum; SEM, scanning electron microscopy.

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were prepared in spectrograde acetone (Aldrich Chemical Co., Milwaukee, Wis.), and phorbol solutions were prepared in normal Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Grand Island Biological Co., Grand Island, N. Y.). All stock solutions were stored at −20° in amber bottles for not more than 3 months.

Cell Culture. C3H/10T1/2 clone 8 cells were used between passages 5 and 12. Procedures for the routine handling and subculturing of the cells have been described previously (29). Except when otherwise indicated, all cells were grown in basal medium Eagle's with 10% heat-inactivated FCS (Grand Island Biological Co.). Some studies were performed with a methylcholanthrene-transformed oncogenic variant of C3H/10T1/2, 58 MCA Cl 16, between passages 29 and 40 (28).

Light Microscopy. For these experiments, 2×10^4 C3H/10T1/2 or 58 MCA Cl 16 cells were plated in 25 sq cm plastic flasks (Corning Glassworks, Corning, N. Y.). At various stages of growth after plating, TPA, acetone, or phorbol was added to each flask by micropipet to result in final concentrations of 0.1 μg of TPA per ml, 0.1 μg of phorbol per ml, and/or 0.2% acetone. The morphology of the cells was then observed at regular intervals, and photographic records were taken on Pantatomic X film (Eastman Kodak Co., Rochester, N. Y.) with a Nikon Model MSS binocular inverted microscope equipped with an AFMB automatic exposure attachment (Nippon Kogaku, Inc., Garden City, N. Y.). Subsequent treatments were performed as indicated.

SEM. SEM studies were carried out in order to ascertain changes not visible by light microscopy. Cultures were set up in dishes with glass coverslips. Two to 4-day-old cultures were treated with TPA (0.1 μg/ml). The cells were examined immediately, 30 min, and 1, 24, 48, and 72 hr after continuous exposure to TPA. Parallel cultures, seeded at the same cell density and exposed to 0.5% acetone for the duration of the TPA exposure, served as controls.

Glass coverslip cultures of cells were fixed and processed *in situ*. After brief rinsing with balanced salt solution, the cultures were fixed at 37° for 30 to 60 min in a mixture of 2% glutaraldehyde and 1% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 1 mM calcium chloride. They were next rinsed in 0.1 M cacodylate buffer containing 0.25 M sucrose and fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 1 to 2 hr at room temperature. Following dehydration in graded series of ethanol and critical-point drying from carbon dioxide, the specimens were sputter coated with gold-palladium prior to SEM examination at an angle of 0 to 45°.

Growth Analysis. The plating efficiency of cells was measured by the colony formation method detailed by Reznikoff *et al.* (28). Briefly, 100 to 200 cells were plated in 60-mm plastic Petri dishes (Corning) in 5 ml of medium. TPA, phorbol, or acetone was added to the cell suspensions just prior to plating. The final concentration of TPA or phorbol in treated cultures was 0.1 μg/ml. Acetone concentrations were always 0.2% in TPA-treated, phorbol-treated, and control dishes. The dishes (4 to 6 per treatment group) were then incubated 8 to 10 days at 37° in a humidified incubator (Forma Scientific Co., Marietta, Ohio) in an atmosphere of 5% CO_2. Dishes were then gently washed with 0.9% NaCl solution, fixed for 10 min in absolute methanol, and stained for 30 min with 5% Giemsa. Colonies were then scored (28) with a Stereozoom dissecting microscope.

The growth rates and saturation densities of the cells were determined by growth curve analysis. In these experiments, 5×10^3 cells were plated in 60-mm dishes in 5 ml of medium. TPA, phorbol, or acetone was introduced to cell suspensions just prior to plating and was included in subsequent biweekly medium changes. Every 24 hr (or longer if growth was judged to be slow), 2 dishes were washed with 0.9% NaCl solution and treated with 2 ml of 0.1% trypsin (Grand Island Biological Co.), and the contents of each dish were separately suspended in 0.9% NaCl solution. Total cell counts for each dish were determined using a Coulter Model B counter (Coulter Electronics, Hialeah, Fla.). Values for duplicate dishes were then averaged and plotted on a graph of log cell number *versus* time.

For data analysis, growth curve lag times were read from the graph. Doubling times were determined by least-squares fit linear regression analysis of the exponential growth phase (linear phase) of each plot. Slopes so obtained were used to calculate doubling times. Saturation densities were determined from the plateau of each growth curve. For C3H/10T1/2 cells, saturation densities were determined by averaging the total cell count values for at least 3 independent sampling times taken more than 48 hr after confluence was reached. For 58 MCA Cl 16 cells, the growth curves showed no true plateaus. Saturation densities were arbitrarily designated as the highest cell number per dish reproducibly reached 10 days after confluence was reached.

Assay for Viability Loss. The viability of C3H/10T1/2 Cl 8 cells in 1% FCS was assayed by a variation of the plating efficiency method described earlier. Dishes (60 mm) were seeded with 200 cells in medium containing 1% FCS. TPA, phorbol, or acetone was introduced just prior to plating. At 24-hr intervals, groups of 6 or 7 dishes were randomly selected from each treatment regimen, changed to medium containing 10% FCS, and incubated 8 to 10 days. The dishes were then fixed and stained. Colonies that developed in the dishes were assumed to be indicative of single cells that had remained viable in 1% serum for the indicated periods of time.

RESULTS

TPA exerted rapid and dramatic effects upon the morphology of C3H/10T1/2 cells. When logarithmically growing cultures were treated with 0.1 μg of TPA per ml (the concentration routinely used for promotion), the cells rapidly rounded up and became highly refractile (Fig. 1). Long beady cytoplasmic processes became evident. Similar effects were seen when confluent cultures were treated. These changes were evident within 30 min of treatment and appeared to reach a maximum at 1 hr. By 24 hr after addition of TPA, these effects had begun to diminish; cells were less round and better spread, and the cytoplasmic processes were less evident. By 72 hr, the cells appeared to be morphologically normal. If fresh TPA was added to cultures 72 hr after the initial treatment, similar effects were seen but were much less pronounced. On a third treatment with TPA 72 hr later, the effects on morphology had diminished to the point where they were no longer evident (data not shown).

Phorbol, which lacks tumor-promoting capability on mouse

skin (12) or on these cells (24), did not exert such morphological effects on the cells, nor were they evident in acetone controls. When the transformed cell line 58 MCA Cl 16 was treated in a similar fashion, the changes were comparable to those seen in C3H/10T1/2 Cl 8 cells (Fig. 2).

SEM confirmed the light microscopy findings. Cultures treated with 0.5% acetone did not show detectable surface changes (Fig. 3*a*). Following TPA treatment, almost all cells showed alterations. At 1 hr, the cells were rounded and retracted from the growth surface. Attachment to the coverslip was only by a few lamellopodia (Fig. 3*b*). Many of the cells examined at 24 hr following TPA treatment appeared to be respreading. However, a significant proportion (10 to 30%) of the cells remained rounded with very few anchorage extensions. At 48 hr following TPA exposure, about 20 to 40% of the cells were partially rounded and incompletely spread (Fig. 3*c*), whereas control cultures were well spread and were confluent by this time. A significant number of cells at 24 and 48 hr following the first TPA treatment showed many microvilli on their surfaces, especially when incompletely spread (Fig. 3*c*). No significant surface changes could be discerned in cultures examined after varying times following a second TPA exposure. The surfaces of TPA-treated cells of later times were generally smooth and comparable to control cells. Substantially the same results were obtained when cells pretreated with an initiating concentration of 3-methylcholanthrene (0.1 μg/ml) were treated with TPA (data not shown).

Before studies of the effects of TPA on C3H/10T1/2 growth properties were initiated, preliminary experiments were conducted to determine the serum requirements of the cells (data not shown). C3H/10T1/2 cells grew well in 10, 5, and 2.5% FCS. The effects of decreasing FCS concentration on cell doubling time were minimal although a progressive decrease in saturation density was observed. However, in 1% FCS, the growth of C3H/10T1/2 cells was inhibited markedly. Long lag times were observed, and the cells grew with doubling times that ranged from 48 to 72 hr or longer. The doubling time of the cells in 1% FCS, but not in 10% FCS, varied dramatically with the serum batch used.

The number of cells seeded per dish was found to be an important factor in the growth rate of C3H/10T1/2 cells. While the number of cells seeded did not significantly affect cell growth in 10% FCS, growth in 1% serum was markedly enhanced when 1×10^4 or more cells were seeded per dish (data not shown). Increasing the number of cells seeded per dish did not affect the saturation density in 1 or 10% FCS. This enhancement of growth with increasing cell density was observed only with serum batches that yielded doubling times of 70 hr or more when 5×10^3 cells were plated per dish. Only 2 of 7 serum batches tested yielded such long doubling times.

This behavior contrasts markedly with that of the transformed 58 MCA Cl 16 cells. These cells grew well in serum concentrations that ranged from 1 to 10% FCS although a reduction in saturation density was observed with decreasing FCS concentration. This decrease was due, at least in part, to increased peeling off of cells from heavily confluent dishes during medium changes. Increased lag times were also evident in 1% FCS. Cell doubling times increased only slightly, from 16 to 24 hr, with decreasing FCS concentration. The variability of cell doubling times with FCS batch was much less pronounced than that observed for the nontransformed cells (data not shown). These results parallel the observations of Bertram (2) on C3H/10T1/2 cell growth as well as the large body of evidence documenting serum requirements for growth in other transformed and nontransformed cell types (6, 16).

When 0.1 μg of TPA or phorbol per ml was included in medium containing 10% FCS, the growth of C3H/10T1/2 cells was not dramatically altered (Table 1). TPA did, however, induce a 20 to 30% increase in saturation density in all serum batches tested (Table 1). An increase in saturation density was not found in an earlier study (27) and was probably masked by normal fluctuations in cell density at confluence. However, increases became apparent with repetition of experiments and statistical treatment of data. A slight increase in the saturation density of C3H/10T1/2 cells following TPA treatment has also been observed by Weinstein *et al.* (32). They found further that TPA induced a 3-fold increase in C3H/10T1/2 saturation density if the cells were grown in Dulbecco's modification of minimal essential medium instead of the Eagle's basal medium used in our studies. The reason for this large TPA-induced increase in saturation density when cells are cultured in Dulbecco's minimal essential medium is not known.

The results were markedly different when the cells were grown in 1% FCS (Chart 1). TPA, but not phorbol, significantly decreased doubling times. TPA also increased the saturation density about 40% (Table 1). The stimulation of growth rate by TPA in 1% FCS was observed only with FCS batches in which the cell doubling times for untreated cells were 70 hr or longer. Only 2 of 7 serum batches tested supported C3H/10T1/2 growth with such long doubling times, and stimulation of growth rate was seen only in these 2 batches. Similarly, stimulation was not observed in even those 2 batches if the initial cell density at the start of an experiment was greater than 5×10^3 cells. Increases in saturation density, however, were observed

Table 1

The effect of TPA and phorbol on the growth properties of 10T1/2 Cl 8 cells

	Property tested							
	Plating efficiency		Saturation density (cells/60-mm dish)		Growth curve lag time (days)		Population doubling times (hr)	
Treatment schedule	10% FCS	1% FCS	10% FCS	1% FCS	10% FCS	1% FCS	10% FCS	1% FCS
Acetone	20.0 ± 0.6[a]	—[b]	$6.0 \times 10^5 \pm 0.4$	$2.9 \times 10^5 \pm 0.2$	2	6–9	15 ± 1	74 ± 6
Phorbol	20.4 ± 0.8	—	$7.5 \times 10^5 \pm 0.7$	$2.9 \times 10^5 \pm 0.2$	2	9	16 ± 2	78 ± 4
TPA	25.5 ± 0.7	—	$1.0 \times 10^6 \pm 0.1$	$4.0 \times 10^5 \pm 0.5$	1–2	2–4	16 ± 1	50 ± 2

[a] Mean ± S.D.
[b] Values vary with number of cells plated (see text).

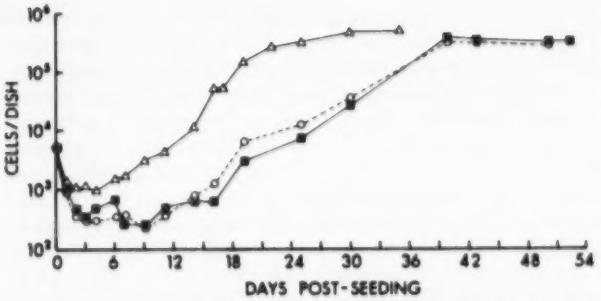

Chart 1. Effect of TPA and phorbol on C3H/10T1/2 cells grown in 1% FCS. Cells were grown in the presence of 0.2% acetone (■), 0.1 μg phorbol per ml (○), or 0.1 μg TPA per ml (△).

in all serum batches tested and at all cell densities plated.

Studies were also conducted to examine the viability of C3H/10T1/2 cells plated in 1% FCS and allowed to incubate for varying periods of time before being changed to medium containing 10% FCS. Since few, if any, colonies resulted when the cells were left to grow in 1% FCS, the colonies obtained after the medium change to 10% FCS were assumed to be indicative of the ability of the cells to survive in 1% FCS. These studies revealed that, in the 2 FCS batches that supported only poor cell growth, C3H/10T1/2 cells remained viable for 3 days in 1% FCS, after which time their viability began to decrease rapidly. As shown in Chart 2, TPA, but not phorbol, facilitated cell survival. In addition, cells plated directly into medium containing 10% FCS showed a slight increase in their plating efficiency when TPA was present (Table 1). Loss of viability in dishes containing 1% FCS could not be restored by refeeding the cells on Day 3 with fresh medium containing 1% FCS. The TPA stimulation of viability could not be attributed to differences in cell adhesion, which might have resulted in the preferential loss of acetone- or phorbol-treated cells in the medium change to 10% FCS. When the experiment was performed by gently adding FCS to the dishes in lieu of a complete medium change, the results were substantially the same as when complete medium changes were performed.

Viability of C3H/10T1/2 cells in 1% FCS medium did not decline as rapidly when the cells were plated into medium containing FCS which supported good (doubling time < 70 hr) cell growth in low serum (data not shown). TPA did not enhance cell viability in such serum batches (data not shown), suggesting that a relationship exists between the ability of TPA to enhance cell viability and cell growth in medium containing 1% FCS.

Attempts were made to determine the plating efficiency of C3H/10T1/2 cells in 1% FCS. Unfortunately, reproducible data could not obtained. C3H/10T1/2 cells had a very low (0.5%) plating efficiency when seeded at low density into medium containing 1% FCS. When the number of cells plated per dish was increased, large increases in plating efficiency resulted. Thus, relatively minor variations in cell seeding density led to dramatic differences in results. While TPA often seemed to increase plating efficiency under these conditions, the effect was exceedingly difficult to quantitate.

Studies were also conducted to examine the effects of TPA and phorbol on the growth of transformed 58 MCA Cl 16 cells. A number of effects were seen, each independent of serum batch. The growth of these transformed cells in 10% FCS was affected by TPA with respect only to saturation density; a 30 to 40% reduction in total cell number per dish was observed in the presence of TPA. Representative growth curves are shown in Chart 3. This decrease in saturation density was not accompanied by unusual amounts of cell detachment.

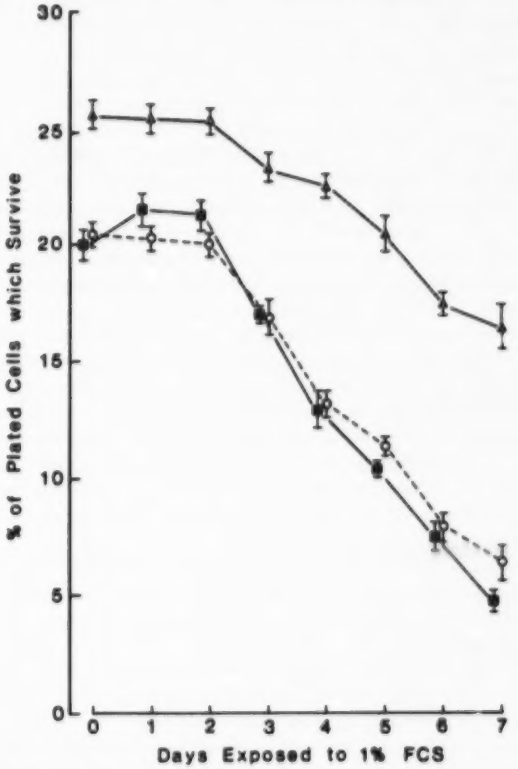

Chart 2. The decline of C3H/10T1/2 cell viability plated at low density in 1% FCS. As described in "Materials and Methods," cells were plated in 0.2% acetone (■), 0.1 μg phorbol per ml (○), and 0.1 μg TPA per ml (△). Each *point*, mean of 3 separate experiments; *bars*, S.D.

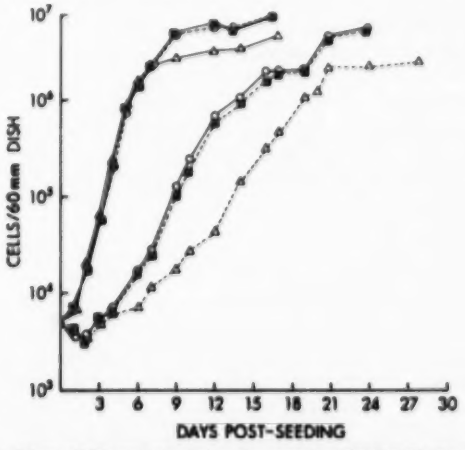

Chart 3. Effect of TPA and phorbol on the growth of 58MCA Cl 16 cells in 1 and 10% FCS. Cells were seeded as described in "Materials and Methods" in the presence of 10% FCS + 0.2% acetone (■——■), 10% FCS + 0.1 μg phorbol per ml (○- - -○), 10% FCS + 0.1 μg TPA per ml (△——△), 1% FCS + 0.2% acetone (■- - -■), 1% FCS + 0.1 μg TPA per ml (△- - -△), and 1% FCS + 0.1 μg phorbol per ml (○——○).

Table 2
The effect of TPA and phorbol on the growth properties of 58MCA Cl 16 cells

Treatment schedule	Property tested							
	Plating efficiency		Saturation density (cells/60-mm dish)		Growth curves lag time (days)		Population doubling times (hr)	
	10% FCS	1% FCS	10% FCS	1% FCS	10% FCS	1% FCS	10% FCS	1% FCS
Acetone	52.5 ± 4.5[a]	9.5 ± 0.1	7.35×10^6	3.49×10^6	1	2	15 ± 1	27 ± 2
Phorbol	50.8 ± 4.5	9.6 ± 0.3	7.37×10^6	3.51×10^6	1	2	15 ± 1	28 ± 2
TPA	47.0 ± 3.7	9.1 ± 0.4	4.68×10^6	2.38×10^6	1	6	15 ± 1	45 ± 3

[a] Mean ± S.D.

The inhibitory effects of TPA were more evident when the cells were treated in medium containing 1% FCS. As shown in Chart 3 and Table 2, the presence of TPA in sparsely seeded cultures resulted in extended lag times, increased doubling times, and lower saturation densities. The effect was relatively independent of serum batch and was seen for one FCS batch in which TPA stimulated the growth of C3H/10T1/2 and in 2 batches where no stimulation was seen. Phorbol did not exert any effects on the growth of transformed 58 MCA Cl 16 cells.

Table 2 summarizes the results obtained in studies using a single FCS batch. In addition to the information obtained in growth curves, studies conducted on the plating efficiency of 58 MCA Cl 16 cells in 10% and 1% FCS failed to detect any significant effects exerted by TPA or phorbol. The inhibition by TPA of 58 MCA Cl 16 cell growth in confluent cultures and in cultures growing in 1% FCS would thus not appear to be accompanied by an effect on cell viability.

DISCUSSION

Studies conducted in several cell culture systems have suggested that the tumor promoter TPA causes nontransformed cells to acquire properties characteristic of the transformed state (3, 7, 9, 33). Similarly, the expression of transformed properties is reported to be enhanced in TPA-treated virus-transformed cells (11, 31, 34). Such observations have led to the hypothesis that phenotypic enhancement of the transformed state is an integral part of the biological mechanisms that underlie tumor promotion (7, 32). Since the phenomenon of promotion has been demonstrated in the C3H/10T1/2 cell culture system, it was of interest to determine whether TPA could cause the acquisition of transformed properties in nontransformed C3H/10T1/2 cells or the enhancement of such properties in chemically transformed C3H/10T1/2 cells.

The choice of properties to be examined in this study was limited because transformed C3H/10T1/2 cells generally do not lose large external transformation-sensitive protein (14), and increases in fibrinolytic activity do not correlate well with transformation (14). Oncogenic transformation is accompanied by anchorage independence (14), but TPA was not able to induce this behavior in C3H/10T1/2 Cl 8 cells.[6] Transformation also alters the morphology (28), cell surface features (21), and growth characteristics (2, 28) of C3H/10T1/2 cells. We have examined the effects of TPA on these properties in transformed and nontransformed C3H/10T1/2 cells. Our results do not support the hypothesis that tumor promoter-induced mimicry of the transformed state bears a mechanistic relationship to promotion.

[6] C. Boreiko and C. Heidelberger, unpublished results.

TPA, but not phorbol, exerted rapid effects upon the morphology of C3H/10T1/2 cells and upon cells of an oncogenically transformed variant of C3H/10T1/2. Both cell types rapidly rounded and became highly refractile when observed by light microscopy. These changes occurred within 30 min in both confluent and logarithmically growing cultures. These TPA-induced changes were similar to those induced by TPA in other cell types (9, 30, 32) and are consistent with alterations in organization of the cytoskeleton known to accompany transformation in other systems (5). SEM confirmed the light microscopy findings and further revealed the transient presence of numerous microvilli on the surface of TPA-treated cells. The observation that TPA induces cell surface alterations could also be interpreted as induction of a transformed phenotype in C3H/10T1/2 cells by TPA. However, the morphological alterations we observed reversed within 72 hr and were substantially diminished in subsequent treatments with TPA. The transient nature of the morphological alterations suggests that their continued expression is not essential for the promotion of C3H/10T1/2 cell transformation. Indeed, C3H/10T1/2 cells appear to acquire a tolerance to the presence of TPA with respect to morphological changes. This tolerance may bear greater significance for the mechanism of promotion than did the initial morphological alterations, especially since a similar escape from TPA inhibition of epidermal growth factor binding to HeLa cells was observed by Lee and Weinstein (18).

TPA was also found to affect the growth of C3H/10T1/2 cells. Treated cultures growing in medium containing 10 or 1% FCS exhibited slight (20 to 40%) increases in saturation density. These increases are minor when compared to those observed with the loss of density-dependent control of cell division which accompanies transformation (28). Nor is this TPA-induced increase in saturation density comparable to the massive hyperplasia induced by tumor promoters on mouse skin (1, 4).

TPA did not significantly alter the growth rate of C3H/10T1/2 cells in medium containing 10% FCS but was found to be capable of markedly decreasing the doubling time of C3H/10T1/2 cells grown in 1% FCS. The ability of C3H/10T1/2 cells to grow in medium containing 1% FCS varied dramatically with the serum batch used; average doubling times for different batches ranged from approximately 50 to 70 hr or longer. Stimulation of C3H/10T1/2 growth by TPA in 1% FCS was only observed in studies utilizing serum batches which yielded doubling times of 70 hr or longer. Two of 7 serum batches that were tested fell into this category.

The ability of TPA to stimulate growth in low serum in select serum batches is perhaps consistent with suggestions that tumor promoters cause cells to acquire transformed character-

istics (3, 7, 9, 33) and can also be likened to the stimulation of DNA synthesis that accompanies promoter-induced hyperplasia *in vivo* (1, 4). The serum batch specificity of this effect, however, argues against the growth enhancement being similar to the general stimulation of growth potential which occurs with transformation. Rather, we suggest that TPA is capable only of relieving growth restrictions imposed by deficiencies in specific serum factor(s) present in limiting concentrations in some FCS batches. TPA may actually reduce C3H/10T1/2 cell requirements for growth, or it may interact with cells in place of specific serum factors and fulfill the role of such factors for growth. In this light, it is interesting to note that TPA enhanced C3H/10T1/2 viability in 1% FCS in a fashion analogous to cell survival factors known to be present in serum (13, 19). Other studies have similarly suggested that TPA can function in a fashion analogous to that of growth factors (8, 32).

In contrast to the occasional stimulation of C3H/10T1/2 cell growth by TPA, the growth of an oncogenically transformed variant of C3H/10T1/2 (58 MCA Cl 16) was found to be inhibited by TPA. TPA reduced the saturation density of the transformed cell line in both 1 and 10% FCS. In addition, the doubling time of sparse cultures of transformed cells in 1% FCS was substantially increased. The growth of transformed cells in sparse cultures in 10% FCS was not inhibited by TPA, and TPA did not cause any detectable decrease in cell viability under any growth condition examined. This growth inhibition would thus appear to constitute an inhibition of phenotypic expression of the transformed state.

The concentration of TPA used in this study (0.1 μg/ml) is also the concentration of TPA which optimally promotes transformation in C3H/10T1/2 cells (24, 25). Our results therefore, suggest that a simple phenotypic mimicry of the transformed state is probably not responsible for promotion in this system. Indeed, the ability to enhance expression of transformed properties in other cell types may be characteristic only of phorbol-related tumor promoters and not of tumor promoters in general (10). Our results therefore stress the importance of choosing appropriate cell culture systems for the study of tumor promotion. Just as TPA has variable effects on differentiation (7, 20, 22) and biochemical responses (7, 26) in different cell types, so TPA may have opposing effects on the expression of the transformed phenotype in cells transformed by viruses or chemicals. Since tumor promotion is generally a phenomenon restricted to chemical or physical carcinogenesis, the effects of tumor promoters should best be evaluated using cells in which initiation and promotion of transformation have been unequivocally demonstrated.

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REFERENCES

1. Berenblum, I. Sequential aspects of chemical carcinogenesis in skin. *In*: F. F. Becker (ed.), Cancer, Vol. 1, pp. 323–344. New York: Plenum Publishing Corp., 1975.
2. Bertram, J. S. Effects of serum concentration on the expression of carcinogen-induced transformation in the C3H/10T1/2 Cl 8 cell line. Cancer Res., *37*: 514–523, 1977.
3. Blumberg, P. M., Driedger, P. E., and Rossow, P. W. Effect of a phorbol ester on a transformation-sensitive surface protein of chick fibroblasts. Nature (Lond.), *264*: 446–447, 1976.
4. Boutwell, R. K. The function and mechanisms of promoters of carcinogenesis. Crit. Rev. Toxicol., *2*: 419–443, 1974.
5. Brinkley, B. R., Miller, C. L., Fuseler, J. W., Pepper, D. A., and Wible, L. J. Cytoskeletal changes in cell transformation to malignancy. *In*: G. F. Saunders (ed.), Cell Differentiation and Neoplasia, pp. 419–450. New York: Raven Press, 1978.
6. Clarke, G. D., Stoker, M. G. P., Ludlow, A., and Thornton, M. Requirement of serum for DNA synthesis in BKH/21 cells: effect of density, suspension, and virus transformation. Nature (Lond.), *227*: 498–501, 1970.
7. Diamond, L., O'Brien, T. G., and Rovera, G. Tumor promoters: effects on proliferation and differentiation of cells in culture. Life Sci., *23*: 1979–1988, 1978.
8. Dicker, P., and Rozengurt, E. Stimulation of DNA synthesis by tumour promoter and pure mitogenic factors. Nature (Lond.), *246*: 723–726, 1978.
9. Driedger, P. E., and Blumberg, P. M. The effect of phorbol diesters on chicken embryo fibroblasts. Cancer Res., *37*: 3257–3265, 1977.
10. Driedger, P. E., and Blumberg, P. M. Non-phorbol mouse skin tumor promoters do not mimic phorbol myristate acetate in its effects on chick embryo fibroblasts. Int. J. Cancer, *22*: 63–69, 1978.
11. Goldfarb, R. H., and Quigley, J. P. Synergistic effect of tumor virus transformation and tumor promoter treatment on the production of plasminogen activator by chick embryo fibroblasts. Cancer Res., *38*: 4601–4609, 1978.
12. Hecker, E. Isolation and characterization of the cocarcinogenic principles from croton oil. *In*: H. Busch (ed.), Methods in Cancer Research, Vol. 6, pp. 439–484. New York: Academic Press, Inc., 1971.
13. Holley, R. W. Control of growth of mammalian cells in cell culture. Nature (Lond.), *258*: 487–490, 1975.
14. Jones, P. A., Laug, W. E., Gardner, A., Nye, C. A., Fink, L. M., and Benedict, W. F. *In vitro* correlates of transformation in C3H/10T1/2 clone 8 mouse cells. Cancer Res., *36*: 2863–2867, 1976.
15. Kennedy, A. R., Mondal, S., Heidelberger, C., and Little, J. B. Enhancement of x-ray transformation by 12-*O*-tetradecanoylphorbol-13-acetate in a cloned line of C3H mouse embryo cells. Cancer Res., *38*: 439–443, 1978.
16. Lasne, C., Gentil, A., and Chouroulinkov, I. Two stage malignant transformation of rat fibroblasts in tissue culture. Nature (Lond.), *247*: 490–491, 1974.
17. Lasne, C., Gentil, A., and Chouroulinkov, I. Two-stage carcinogenesis with rat embryo cells in tissue culture. Br. J. Cancer, *35*: 722–729, 1977.
18. Lee, L-S., and Weinstein, I. B. Mechanism of tumor promoter inhibition of cellular binding of epidermal growth factor. Proc. Natl. Acad. Sci. U. S. A., *76*: 5168–5172, 1979.
19. Lipton, A., Paul, D., Henahan, M., Kinger, I., and Holley, R. W. Serum requirements for the survival of 3T3 and SV3T3 fibroblasts. Exp. Cell Res., *74*: 466–470, 1972.
20. Lotem J. and Sachs, L. Regulation of normal differentiation in mouse and human myeloid leukemic cells by phorbol esters and the mechanism of tumor promotion. Proc. Natl. Acad. U. S. A. *76*: 5158–5162, 1979.
21. Malick, L., and Langenbach, R. Scanning electron microscopy of *in vitro* chemically transformed mouse embryo cells. J. Cell Biol., *68*: 654–664, 1976.
22. Miao, R. M., Fieldsteel, A. H., and Fodge, D. W. Opposing effects of tumour promoters on erythroid differentiation. Nature (Lond.), *274*: 271–272, 1978.
23. Mondal, S. The C3H/10T1/2 Cl 8 mouse embryo cell line: its use for the study of carcinogenesis and tumor promotion in cell culture. *In*: M. A. Mehlman, N. K. Mishra, and V. Dunkel (eds.), Advances in Environmental Toxicology, Vol. 1, Princeton Junction: Senate Press, Inc., 1980.
24. Mondal, S., Brankow, D. W., and Heidelberger, C. Two-stage chemical oncogenesis in cultures of C3H/10T1/2 cells. Cancer Res., *36*: 2254–2260, 1976.
25. Mondal, S., and Heidelberger, C. Transformation of C3H/10T1/2 Cl 8 mouse embryo fibroblasts by ultraviolet radiation and a phorbol ester. Nature (Lond.), *260*: 710–711, 1976.
26. O'Brien, T. G., Lewis, M. A., and Diamond, L. Ornithine decarboxylase activity and DNA synthesis after treatment of cells in culture with 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res., *39*: 4477–4480, 1979.
27. Peterson, A. R., Mondal, S., Brankow, D. W., Thon, W., and Heidelberger, C. Effects of promoters on DNA synthesis in C3H/10T1/2 mouse fibroblasts. Cancer Res., *37*: 3223–3227, 1977.
28. Reznikoff, C. A., Bertram, J. S., Brankow, D. W., and Heidelberger, C. Quantitative and qualitative studies of chemical transformation of clones of C3H mouse embryo cells sensitive to post-confluence inhibition of cell division. Cancer Res., *33*: 3239–3249, 1973.
29. Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to post-confluence inhibition of cell division. Cancer Res., *33*: 3231–3238, 1973.
30. Sivak, A. Induction of cell division in BALB/c-3T3 cells by phorbol myristate acetate or bovine serum: effects of inhibitors of cyclic AMP phosphodiesterase and Na^+-K^+ATPase. In Vitro (Rockville), *13*: 337–343, 1977.
31. Sivak, A., and Van Duuren, B. L. A cell culture system for the assessment of tumor-promoting activity. J. Natl. Cancer Inst., *44*: 1091–1097, 1970.
32. Weinstein, I. B., Wigler, M., Fisher, P. B., Sisskin, E., and Pietropaolo, C.

Cell culture studies on the biologic effects of tumor promoters. In: T. S. Slaga, A. Sivak, and R. K. Boutwell (eds.), Carcinogenesis: Mechanisms of Tumor Promotion and Cocarcinogenesis, Vol. 2, 313–334, 1978.
33. Wigler, M., and Weinstein, I. B. Tumor promoter induces plasminogen activator. Nature (Lond.), 259: 232–233, 1976.
34. Wilson, E. L., and Reich, E. Plasminogen activator in chick fibroblasts: induction of synthesis by retinoic acid; synergism with viral transformation and phorbol ester. Cell, 15: 385–392, 1978.

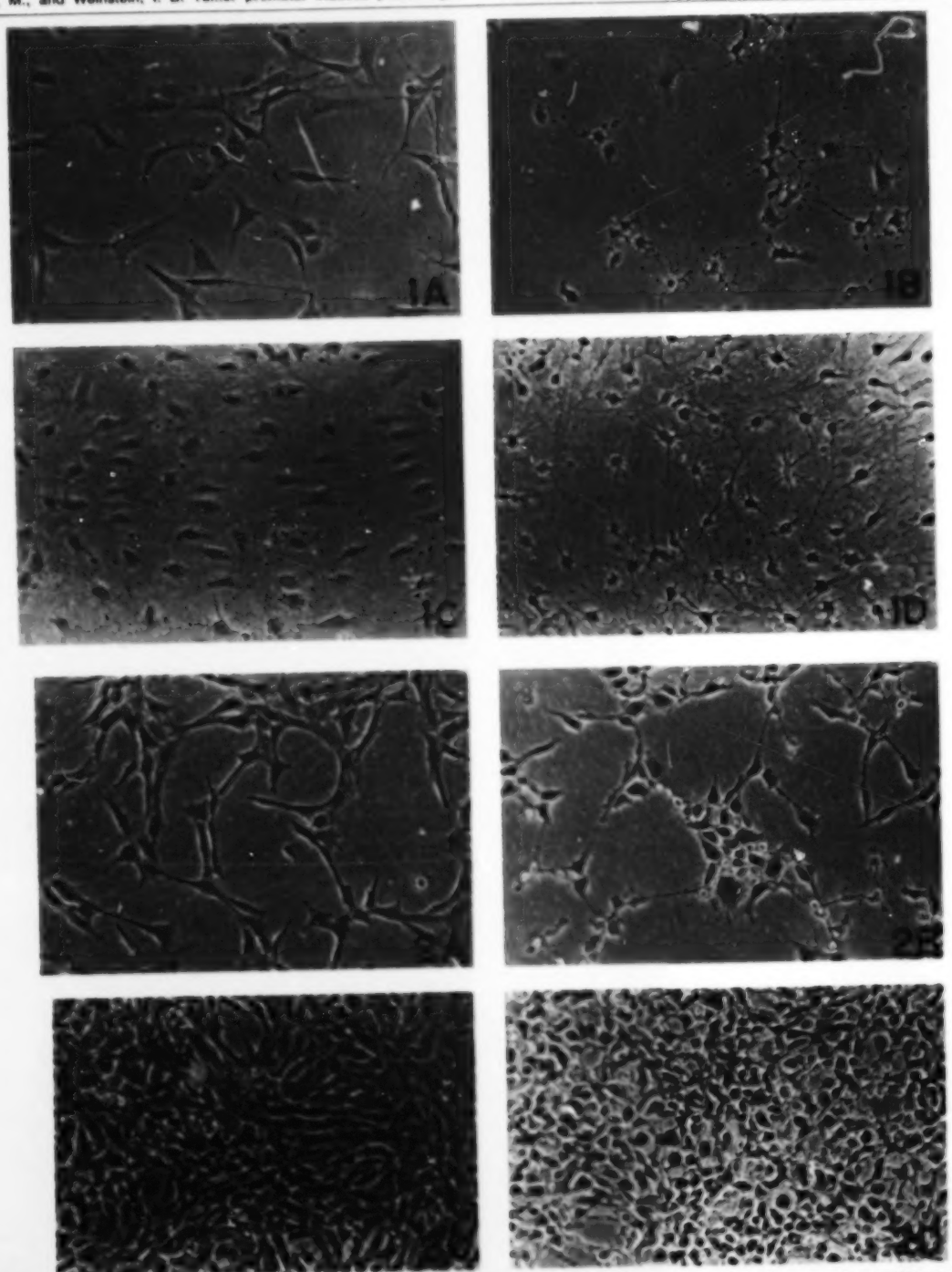
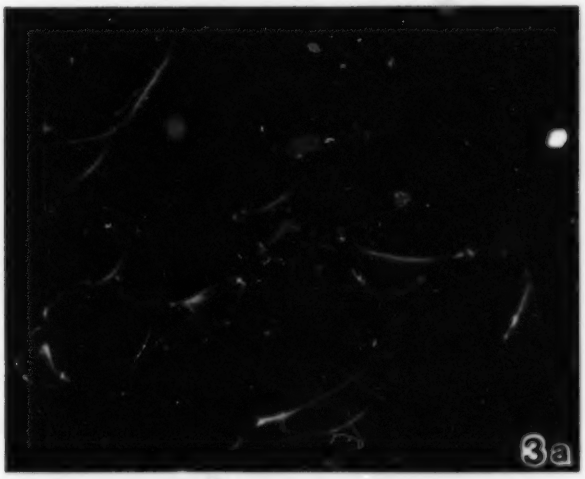
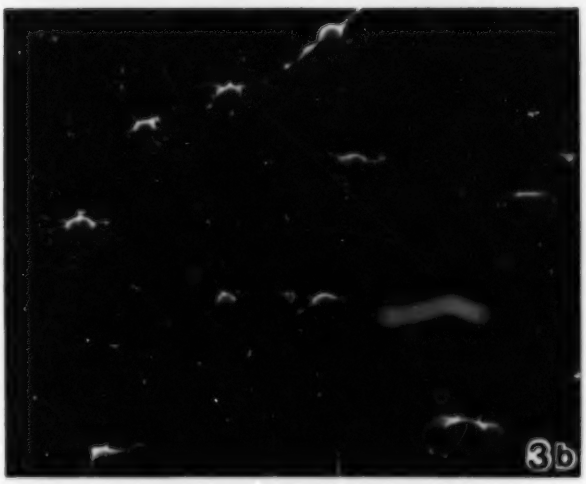


Fig. 1. Phase-contrast photomicrographs. *A*, logarithmically growing C3H/10T1/2 cells after 1 hr of exposure to 0.2% acetone in complete medium. × 300. *B*, logarithmically growing C3H/10T1/2 cells after 1 hr of exposure to 0.1 μg TPA per ml in complete medium. × 300. *C*, confluent C3H/10T1/2 culture 1-hr after treatment with 0.2% acetone. × 300. *D*, confluent C3H/10T1/2 culture 1 hr after treatment with 0.1 μg TPA per ml. × 300.

Fig. 2. Phase-contrast photomicrographs. *A*, logarithmically growing 58MCA Cl 16 cells after 1 hr of exposure to 0.2% acetone in complete medium. × 300. *B*, logarithmically growing 58MCA Cl 16 cells after 1 hr of exposure to 0.1 μg TPA per ml in complete medium. × 300. *C*, confluent 58MCA Cl 16 culture 1 hr after treatment with 0.2% acetone. × 300. *D*, confluent 58MCA Cl 16 culture 1 hr after treatment with 0.1 μg TPA per ml. × 300.





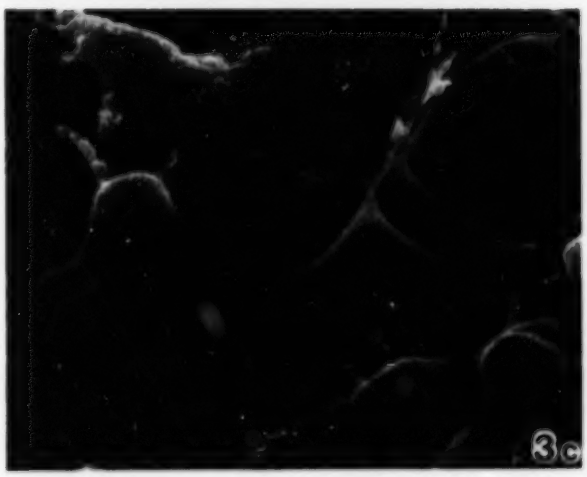


Fig. 3. SEM's of C3H/10T1/2 cells demonstrating changes following TPA treatment. *a*, sparse culture of control cells 3 days after seeding. × 200. *b*, sister culture fixed immediately following 1-hr exposure to TPA. × 200. *c*, 5-day-old culture 48 hr after exposure to TPA. Many of the cells are still only partially spread and demonstrate long beady cytoplasmic processes and numerous microvilli. × 1000.